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http://dx.doi.org/10.1289/ehp.1205188

Online 24 September 2012

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National Institute of Environmental Health Sciences

National Institutes of Health
U.S. Department of Health and Human Services

Hyaluronan Activation of the Nlrp3 Inflammasome Contributes to the Development of Airway Hyperresponsiveness

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Running Title: Nlrp3 inflammasome and airways disease

Key Words: asthma, environment, extracellular matrix, innate immunity, ozone, toll-like receptor

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Acknowledgements: This work was supported by National Institutes of Health grants ES016126, AI081672, ES020350 & ES020426 (to JWH), AI089756 (to KLW), and an unrestricted educational grant from the China Scholarship Council (to FF).

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Competing Financial Interests: None of the authors has any actual or potential competing financial interests.

Abbreviations:

AHR: Airway hyperresponsiveness

BAL: Bronchoalveolar lavage fluid

FA: Filtered air

HA: Hyaluronan fragments, low molecular weight hyaluronan

HABP: Hyaluronic acid-binding peptide

HMW-HA: High molecular weight hyaluronan

SBP: Scrambled-binding peptide

WT**:** Wild-type

Abstract

Background: The role of the Nlrp3-inflammasome in non-allergic airway hyperresponsiveness (AHR) has not previously been reported. Recent evidence supports that both IL-1β and short fragments of hyaluronan (HA) contribute to the biological response to inhaled ozone.

Objectives: Because extracellular secretion of IL-1β requires activation of the inflammasome, we investigated the role of the inflammasome proteins ASC, caspase1, and Nlrp3 in the biological response to ozone and hyaluronan.

Methods: C57BL/6J, ASC-/-, caspase1-/- and Nlrp3-/- mice were exposed to ozone (1 ppm x 3 hrs) or HA followed by analysis of airway resistance, cellular inflammation, total protein and cytokines in bronchoalveolar lavage (BAL). The transcription levels of *IL-1β* and *IL-18* in two populations of lung macrophages were determined. As a marker of inflammasome activation, the level of cleaved caspase1 and cleaved IL-1β were examined in isolated alveolar macrophages harvested from BAL after mice were treated with HA.

Results: Genes of the Nlrp3-inflammasome are required for development of AHR following exposure to either ozone or HA fragments. Genes of the Nlrp3-inflammasome are partially required for the cellular inflammatory response to ozone. The mRNA expression of *IL-1β* in alveolar macrophages was upregulated after either ozone or HA challenge and was not dependent on Nlrp3 inflammasome. The soluble levels of IL-1β are dependent on the inflammasome after challenge to either ozone or HA. Hyaluronan challenge resulted in cleavage of macrophage-derived caspase1 and IL-1β suggesting a role for alveolar macrophages in Nlrp3-dependent AHR.

Conclusions: The Nlrp3-inflammasome is required for the development of ozone-induced reactive airways disease.

Introduction

Ambient ozone is a highly reactive oxidant associated with increased morbidity and mortality in human populations (Bell et al. 2004; Bell et al. 2006; Ito et al. 2005). Despite effective regulatory oversight to reduce ambient levels of ozone (Samet 2011), current evidence support adverse health effects below current regulatory standards (Berman 2012; Kim et al. 2011). Furthermore, it is anticipated that changes in global climate could increase both ambient levels of ozone and associated adverse health effects (Chang et al. 2010). Understanding the mechanisms that regulate the biological response to ozone could have important implications to human health. Inhalation of ozone can immediately cause lung injury, inflammation, and decrements in lung function (Foster et al. 2000; Kehrl et al. 1987; Koren et al. 1989). The delayed responses to ozone include induction of asthma-like phenotypes with compromises in epithelial barrier function and development of airway hyperresponsiveness (AHR) (Que et al. 2011). For this reason, we believe that fundamental insight into the mechanisms that regulate the biological response to ozone could have broad implications to our understanding of the pathogenesis of airways diseases including both asthma and chronic obstructive pulmonary disease (COPD).

Recent evidence support the biological response to ozone is dependent on activation of the innate immune system [reviewed, (Al-Hegelan et al. 2011; Peden 2011)]. It is now recognized that components of the response to ozone requires activation of the surface receptor toll-like receptor 4 (TLR4) (Hollingsworth et al. 2004; Kleeberger et al. 2001; Williams et al. 2007). More recent data has demonstrated that ozone induced production of short fragments of hyaluronan (Garantziotis et al. 2009) activate the TLR4-CD44 surface receptor complex and

contribute to AHR (Garantziotis et al. 2010; Taylor et al. 2007). Interestingly, short fragments of hyaluronan are also detected in the bronchoalveolar lavage (BAL) fluid from patients with both COPD (Dentener et al. 2005) and allergic asthma (Liang et al. 2011; Sahu and Lynn 1978). In our previous studies, the response to either ozone or hyaluronan fragments were also closely associated with the levels of IL-1β in the BAL (Garantziotis et al. 2010; Li et al. 2011). Previous work supports a central role for IL-1 dependent signaling in the biological response to ozone (Johnston et al. 2007; Park et al. 2004; Verhein et al. 2008; Wu et al. 2008). While the specific mechanism by which IL-1β contributes to AHR remains unknown, IL-1β is recognized to increase both cyclooxygenase (COX)-2 expression and prostaglandin E_2 resulting in decreased β-adrenergic responsiveness of smooth muscle cells providing a link to AHR (Moore et al. 2001). However, the mechanisms leading to IL-1β activation in context of either ozone or reactive airways disease remains poorly understood.

The inflammasome protein complex is an important regulator of IL-1β and IL-18 maturation and release into the extracellular space [reviewed, (Hoffman and Brydges 2011; Jin and Flavell 2010)]. The inflammasome complex consists of the protein pro-caspase1 and may include the adaptor molecule ASC, as well as, specific members of the nucleotide-binding domain leucine-rich repeat-containing (NLR) family. Inflammasome activation leads to caspase1 mediated cleavage and activation of the proinflammatory cytokines pro-IL-1β and pro-IL-18. Yamasaki *et al* recently demonstrate that hyaluronan-induced IL-1β release is dependent on the Nlrp3-inflammasome in a model of sterile skin injury (Yamasaki et al. 2009). Recent work in macrophages demonstrates oxidant stress can prime the Nlrp3-inflammasome (Bauernfeind et al. 2011). On the basis of these previous observations and the recognized role of

hyaluronan in the response to ozone, we hypothesized that hyaluronan activation of the Nlrp3-inflammasome would contribute to the airway response to ozone.

Materials and Methods

Mice. Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Caspase1-/- were generously provided by Dr. Fayyaz Sutterwala and Dr. Richard Flavel (Sutterwala et al. 2006). ASC-/- and Nlrp3-/- mice were generously provided from Genentech Inc. (San Francisco, CA). 6-8 week old male mice were used in experiments. Animals were treated humanely with due consideration to alleviation of distress and discomfort. Animal experimental protocols were approved by the Animal Care Committee at Duke University Medical Center and performed in accordance with the standards established by the U.S. Animal Welfare Acts.

Ozone exposure. Mice were exposed in a Hinner-style chamber to either filtered air (FA) or ozone (1 ppm x 3 h) as previously reported (Hollingsworth et al. 2004). Wild type mice were phenotyped 3, 6, 12, 24, 48 and 72 hours after ozone exposure. The time point of 24 hours after exposure was chosen to observe phenotype of C57BL/6J and inflammasome deficient mice based on the level of cellular inflammation, hyaluronan in BAL and the transcription level of IL-1β. Our selection of ozone concentration levels in the mouse is based on similar biological response observed in human exposure studies and published deposition fraction data for ozone in rodent models (Hatch et al. 1994; Wiester et al. 1988). The ozone concentration in the chamber was monitored continuously by a UV light photometer (model 400E; Teledyne Technologies Inc., Thousand Oaks, CA). In blocking experiments, C57BL/6J mice were anesthetized with isoflurane and then 0.22 mg hyaluronic acid-binding peptide (HABP) or scrambled-binding

peptide (SBP) (GenScript, Piscataway,NJ) were oropharyngeally instilled into the lung immediately prior to ozone exposure.

HA challenge. Sterile, endotoxin-free high molecular weight hyaluronan (HMW-HA) (Healon GV; Abbott Medical Optics, Santa Ana, CA) was reconstituted at 0.5 mg/ml in 0.02 M acetate, 0.15 M sodium chloride, pH 6.0. For the production of low molecular weight hyaluronan (HA), HMW-HA was sonicated 3s for three times on ice. Sizes of HA were confirmed by agarose gel electrophoresis. For *in vivo* experiments, 50 µl of HA (25 µg/mouse) or vehicle were instilled intratracheally into isoflurane-anesthetized mice. HA at such a dose was sufficient to induce significantly increased AHR (Garantziotis et al. 2009). Similarly, AHR was measured invasively 2h later. The selection of HA treatment time point was based on HA time course experiment.

Airway Physiology. Direct measurements of airway response to methacholine were performed as previously reported (Garantziotis et al. 2009). Briefly, mice were ventilated with a computer-controlled small animal ventilator (FlexiVent; SCIREQ, Montreal, Quebec, Canada) and measurements of respiratory mechanics were made by the forced oscillation technique. Mice were challenged with aerosolized methacholine (DeVilbiss ultrasonic) at 0, 10, 25 and 100 mg/ml, and airway response was determined by resistance measurements every 30 s for 5 min. Total lung resistance measurements were averaged at each does and graphed ($R_T$cmH_2O/ml/s) along with the initial baseline measurement.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was performed as described previously (Garantziotis et al. 2009). Immediately after pulmonary function measurements, mice were euthanized with CO_2 and the lungs were exposed and inflated fully and serially to 25 cmH_2O of

0.9% NaCl for three times. Cell counting was performed using a hemocytometer and differentials were performed by H&E-stained cytospins. Cytokines/chemokines (IL-1α, IL-1β, IL-6, KC, MCP-1, IL-17 and TNF-α) were determined by Luminex (Bio-Rad, Hercules,CA) using reagents from Millipore (Billerica, MA). IL-18 and C3a were detected by ELISA (Bender MedSystems, Vienna, Austria; Kamiya Biomedical Company, Seattle, WA). Total protein concentration in lung lavage fluid was detected by Lowry Assay (Bio-Rad), HA level in lung lavage fluid was measured by ELISA (Echelon, Salt Lake City, UT).

Alveolar macrophage isolation. Alveolar macrophages were harvested by bronchoalveolar lavage with 0.9% NaCl / 0.5 mM ethylenediamine tetraacetic acid (EDTA) buffer. Briefly, a total of 10 fills of lavage fluid was collected. After centrifugation at 1500 rpm for 10 min, the cell pellet was obtained for RNA analysis. We achieve 98% macrophages with this technique as detected by cytospin.

Whole lung macrophage isolation. After airway physiology measurements and BAL, the lungs were collected from the mouse and placed into a tissue culture dish. They were minced and digested in HBSS with 1 mg/mL collagenase A (Sigma) and 0.2 mg/mL of DNase1 (Sigma) for at 37°C for 1h. The digestion solution was passed through a 70-μm mesh strainer and centrifuged at 1600 rpm, for 20 min at room temperature over an 18% nycodenz (Accurate Chemical Co) cushion. After red blood cell lysis, low density cells were collected and then washed twice. We achieve 87% macrophages by cytospin using this technique. The cell pellet was obtained for RNA analysis.

Real-time PCR. Total RNA was isolated using RNAeasy kit (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase treatment and removal reagents to remove DNA

contamination (Ambion Inc., Austin, TX, USA). RNA samples were then reverse-transcribed into cDNA using SuperScript II RT (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's protocol. Real-time PCRs were performed with the SYBR green detection system (Applied Biosystems, Foster City, CA, USA). Primer sequences used for *IL-1* and *IL-18* amplification are as following: *IL-1β* forward: 5'-CTGTGTCTT TCCCGTGGACC-3', reverse 5'-CAGCTCATATGGGTCCGACA-3'; *IL-18* forward: 5'-CACATGCGCCTTGTGATGAC-3', reverse 5'-TGCAGCCTCGGGTATTCTG-3'; 18s forward: 5'-GCTGCTGGCACCAGACTT-3', reverse 5'-CGGCTACCACATCCAAGG-3'. After ΔΔCt calculation, all results were normalized to 18s ribosomal RNA internal controls and expressed as RNA expression fold increase by comparing with control groups.

Immunoblot analysis. Lavage fluid from 5 mice per group was pooled together as one sample. Alveolar macrophages cell pellets were then centrifuged and dissolved in RIPA lysis buffer with proteinase inhibitors, protein concentration was determined by DC protein assay (Bio-Rad, Hercules, CA), 20 μg protein per sample was mixed with sample loading buffer and boiled for 5 min. Samples were separated by 4~20% SDS-PAGE (Bio-Rad, Hercules, CA) and were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Immunoblots were blocked by 5% milk in TBS/0.1% Tween 20 for 1 hour at room temperature, then incubated overnight at 4 °C with 1:1000 rabbit polyclonal antibody to mouse caspase1 p10 (Santa Cruz, CA) or 1:2000 goat polyclonal antibody to mouse IL-1β (R&D, MN) or 1:1000 goat polyclonal antibody to mouse GAPDH (Santa Cruz, CA) in 3% milk/ TBS/0.1% Tween 20, 1:2000 goat anti-rabbit IgG or 1:2000 donkey anti-goat IgG as secondary antibodies in 3% milk/

TBS/0.1% Tween 20 was used to incubate membranes at room temperature for 1 hour. Bands were detected by ECL plus western blotting detection system (GE Healthcare, Piscataway, NJ).

Statistics. All presented data are representative of at least two replicate experiments. Data are expressed as mean ± SEM, GraphPad (SanDiego, CA) was used for statistical analysis. Significant difference among multiple groups with one variant was determined by one-way ANOVA, every two groups were then compared by Neuman-Keuls. The Student *t* test was used for comparisons between two groups. A two-tailed p value < 0.05 was considered statistically significant.

Results

Significant biological response in the lung is observed 24 hours after ozone inhalation.

We examined the airway response to ozone (1 ppm x 3h) in wild-type mice over a time course of 72 hours. There was a significant increase in total inflammatory cell numbers in BAL from wild-type mice over a time course from 24h to 72h after ozone exposure (Figure 1A). BAL differential cell counts revealed an increase in the numbers of macrophages and neutrophils (Figure 1B-C). The cellular inflammation peaked at 24h after ozone exposure and maintained a high level until 72h. The level of BAL protein peaked from 12h to 48h and declined to close to baseline at 72h after exposure (Figure 1D). We detected BAL hyaluronan levels to examine extracellular matrix injury during ozone-induced acute airway inflammation. The level of hyaluronan in BAL increased at 24h, peaked at 48h, and declined at 72h after ozone exposure (Figure 1E). We examined the transcription level of *IL-1β* and *IL-18* and found that *IL-1β*

mRNA in alveolar macrophages was upregulated at 6h and this level was maintained throughout the 72h time course (Figure 1F). Alveolar macrophages expression of *IL-18* mRNA was increased at 24h and peaked at 72h after exposure (Figure 1G). Based previous epidemiological data and the biological time course, we selected the 24-hour time point after ozone exposure to conduct subsequent studies.

The Nlrp3-inflammasome regulates the inflammatory and AHR response to ozone.

We challenged wild-type C57BL/6J mice (WT) and mice deficient in Nlrp3, caspase1, or ASC either to filtered air (FA) or ozone (1 ppm x 3h). 24h later, we identified that WT mice exposed to ozone developed enhanced sensitivity to methacholine when compared to air control-exposed animals. Animals deficient in ASC, caspase1, or Nlrp3 were completely protected from enhanced AHR after inhalation of ozone and were no different than air-exposed WT animals (Figure 2A). We characterized the level of inflammatory cell influx into the airspace after ozone exposure in each mouse strain (Figure 2B). As anticipated, we observed that the number of total cells, macrophages, and neutrophils in BAL from WT mice after ozone exposure were increased when compared to air-exposed animals. Animals deficient in ASC, caspase1, or Nlrp3 each demonstrated significantly reduced numbers of macrophages and neutrophils, when compared to ozone-exposed WT animals. Next, we measured the level of total protein in the BAL as a marker of lung injury after ozone inhalation. Inflammasome deficient animals had significantly lower levels of total protein in the BAL, when compared to WT mice when exposed to ozone (Figure 2C). To measure cytokine expression directly regulated by the Nlrp3-inflammasome in response to ozone inhalation, we measured transcription levels in alveolar macrophages and BAL soluble levels of IL-1β and IL-18, and observed that the mRNA levels of *IL-1β* and *IL-18*

in alveolar macrophages was upregulated 24h after ozone exposure compared with air controls and was not dependent on Nlrp3-inflammasome **(**Figure 3A,B). Additionally, no differences in mRNA expression level of *IL-1β* and *IL-18* from whole lung macrophages after ozone exposure was observed (Supplemental Material, Figure S1A-B). The levels of secreted IL-1β and IL-18 protein in the BAL from ozone-exposed WT mice were increased and significantly higher than those in BAL from ozone-exposed ASC-/-, caspase1-/-, and Nlrp3-/- mice (Figure 3C, D). We also observed a broad impact of inflammasome activation on additional pro-inflammatory cytokines and mediators previously associated with the biological response to ozone (Supplemental Material, Figure S2A-G). Animals deficient in ASC, caspase1, and to a lesser extent Nlrp3 had reduced levels of IL-1α, IL-6, tumor necrosis factor-α (TNF-α), and KC (cytokine-induced neutrophil chemo-attractant) in BAL after ozone exposure, when compared to WT animals; monocyte chemoattractant protein-1 (MCP-1) was completely dependent on Nlrp3 inflammasome; IL-17 was completely dependent on caspase1, Nlrp3, and partially dependent on ASC; C3a was partially dependent on Nlrp3-inflammasome.

Hyaluronan contributes to the increased level of secreted IL-1β in BAL after ozone exposure.

Similar to observations in wild-type animals, the levels of soluble hyaluronan in BAL from ASC, caspase1, and Nlrp3 deficient animals were increased at 24h after inhalation of ozone. No significant difference was detected when compared with ozone-exposed WT animals (Figure 3E). To further identify the role of HA in ozone-induced inflammasome activation, we pre-treated wild-type animals with hyaluronic acid-binding peptide (HABP), which reduce the level of HA in the lungs (Garantziotis et al. 2009) prior to ozone exposure, and then measured the level of secreted IL-1β in BAL. We found that HABP pretreatment partially blocked the

production of BAL IL-1β (Figure 3F), which support that HA contributes to ozone-induced release of soluble IL-1β.

Significant biological response in the lung is observed after direct hyaluronan challenge.

As previously reported to be functionally relevant (Garantziotis et al. 2009), HA (25 μg/mouse) or vehicle was instilled intratracheally into isoflurane anesthetized wild-type mice. We then characterized the biological responses at 1, 2 and 6h after HA challenge. We found that there was no increase BAL total cell, macrophages or neutrophil numbers until 6h after challenge (Figure 4A-C). No increased BAL protein was detected after HA challenge compared with vehicle at all three time points (Figure 4D). However, *IL-1β* mRNA in alveolar macrophages was upregulated at both 1h and 2h after HA challenge compared with vehicle, and reduced to close to baseline at 6h (Figure 4E). There was no significant difference in the transcription level of *IL-18* after HA challenge compared with vehicle at three time points (Figure 4F). The inflammatory cell influx increased at 6h after challenge, based on the data of *IL-1β* mRNA and the physiological response (Garantziotis et al. 2009; Garantziotis et al. 2010; Li et al. 2011), we selected 2h after HA challenge for subsequent studies.

Nlrp3-inflammasome is required for the pulmonary response to hyaluronan fragments.

To determine the role of hyaluronan activation of the inflammasome in reactive airways disease, we directly challenged animals to short fragments of hyaluronan. As previously reported (Garantziotis et al. 2009; Garantziotis et al. 2010; Li et al. 2011), WT animals developed AHR 2h after challenge to hyaluronan but not vehicle (Figure 5). We observed that the physiological response, as measured by methacholine sensitivity after hyaluronan fragments, was completely

dependent on ASC, caspase1, and Nlrp3. However, similar to WT animals, HA challenge in ASC, caspase1 and Nlrp3 deficient mice had no effect on either the number of cells in the airspace (Supplemental Material, Figure S3A) or the level of total protein in the BAL (Supplemental Material, Figure S3B) at this time point. Similar to the observations after ozone exposure, after HA treatment, *IL-1β* mRNA in alveolar macrophages was upregulated in a manner independent of the Nlrp3-inflammasome (Figure 6A) and the release of soluble IL-1β was completely dependent on ASC, caspase1, and partially dependent on Nlrp3 (Figure 6C). However, we did not observe detectible increases in *IL-18* mRNA expression in alveolar macrophages or soluble IL-18 in BAL after direct challenge to HA (Figure 6B, 6D**).** The increasing of *IL-1β* and *IL-18* mRNA by HA challenge was not detected in whole lung macrophages (Supplemental Material, Figure S1C-D). Moreover, the release of IL-1α, IL-6, MCP-1, TNF- α and KC were dependent on both ASC and caspase1 and partially dependent on Nlrp3; IL-17 was partially dependent on ASC and caspase1, but not dependent of Nlrp3. C3a was dependent on ASC and caspase1 and partially dependent on Nlrp3 after hyaluronan fragment challenge (Supplemental Material, Figure S4A-G).

Hyaluronan activation of the Nlrp3-inflammasome results in cleavage of macrophage-derived caspase1 and IL-1β.

Activation of the inflammasome requires cleavage of pro-caspase1 to an active form that can subsequently cleave pro-IL-1β to an active soluble form. We therefore determined whether *in vivo* challenge to hyaluronan fragments resulted in cleavage of pro-caspase1 in alveolar macrophages. We identified in WT mice that hyaluronan resulted in cleavage of pro-caspase1, which was not observed in ASC-/-, Nlrp3-/-, or caspase1-/- mice (Figure 6E). The cleavage of

pro-IL-1β is resultant from Nlrp3-inflammasome activation. We additionally observe cleavage of pro-IL-1β in alveolar macrophages from WT mice, not from Nlrp3-inflammasome deficient mice (Figure 6F).

Discussion

In support of our hypothesis, we found a dominant role of the Nlrp3-inflammasome in non-allergic reactive airways disease after inhalation of ozone. Development of ozone-induced AHR requires several components of the inflammasome complex including; ASC, caspase1, and Nlrp3. Ozone inhalation results in increased level of soluble hyaluronan, which we have previously reported to contribute to AHR (Garantziotis et al. 2009). We now report that hyaluronan-induced cleavage of alveolar macrophage-derived pro-IL-1β is dependent on the Nlrp3-inflammasome. In addition to IL-1β, the level of many pro-inflammatory factors (IL-1α, IL-6, KC, TNF-α, MCP-1, IL-17, and C3a) are partially dependent on genes of the Nlrp3-inflammasome after challenge to either ozone or HA. Together these novel findings strongly support biological and functional roles of the Nlrp3-inflammasome in the development of ozone-induced reactive airways disease.

Current evidence support an important role for IL-1-dependent signaling in the pulmonary response to ozone (Johnston et al. 2007; Park et al. 2004; Verhein et al. 2008; Wu et al. 2008). Our previous work demonstrated that ozone exposure induced the release of hyaluronan fragments (Garantziotis et al. 2009; Garantziotis et al. 2010) and led to an increase of soluble IL-1β within the airways (Garantziotis et al. 2010; Li et al. 2011). We now recognize from a dermal

injury model that activation of pro-IL-1β is regulated by the Nlrp3-inflammasome in response to hyaluronan (Yamasaki et al. 2009). In the present study, we demonstrate that ASC, caspase1, and Nlrp3 contribute to ozone-induced release of soluble IL-1β.

Although immuno-stimulatory short fragments of hyaluronan can contribute to the production of many macrophage-derived pro-inflammatory cytokines (Garantziotis et al. 2009; Garantziotis et al. 2010), we now identify the relationship with genes of the Nlrp3-inflammasome. The level of soluble hyaluronan in the airspace and macrophage-derived transcript of *pro-IL-1β* was not dependent of ASC, caspase1, or Nlrp3. However, soluble hyaluronan functionally contributes to the generation of active soluble IL-1β after exposure to ozone as observed in hyaluronan-binding experiments. The functional response to direct hyaluronan fragment challenge in the lung was dependent on genes of the inflammasome. We observe a dominant role for both ASC and caspase1 in induction of many pro-inflammatory factors previously associated with the biological response to ozone, including IL-1β, IL-18, IL-1α, IL-6, MCP-1, TNF-α, KC, IL-17, and C3a. These data support that the inflammasome components not only activate IL-1β and IL-18, but can also regulate the release of additional pro-inflammatory cytokines (Barker et al. 2011). The near complete dependence on caspase1 supports activation of the inflammasome and essentially excludes the contribution of pyronecrosis, which is caspase1-independent, but Nlrp3 and ASC dependent (Ting et al. 2008). However, we did not observe consistent complete reduction in any single soluble factor to explain the complete dependence of the Nlrp3-inflammasome in AHR. This observation suggests that factors in addition to measured cytokines may contribute to AHR in the Nlrp3-deficient mouse including additional soluble factors (Backus et al. 2010; Williams et al. 2008), neural responses (Caceres et al. 2009), or smooth muscle function. Furthermore, the partial

reduction in pro-inflammatory cytokines observed in Nlrp3-deficient mice suggests that additional proteins in the NLR family may contribute to the response to both ozone and hyaluronan. While ASC and caspase1 are ubiquitously expressed in many cell types, Nlrp3 is primarily expressed in myeloid cells (Guarda et al. 2011). NLR proteins expressed in non-myeloid cells may prove important. However, the partial dependence on Nlrp3 does suggest an important contribution of myeloid-derived activation of the inflammasome for the complete response to either ozone or hyaluronan. These findings demonstrate that both ASC and caspase1 contribute a dominant role in airway hyperresponsiveness and suggest a functional role for the alveolar macrophages as an important source of pro-inflammatory cytokines.

Current evidence supports an emerging paradigm, in which, inhalation of ozone results in oxidant-induced fragmentation of extracellular matrix hyaluronan (Li et al. 2010). These immune-stimulatory fragments of hyaluronan interact with a surface receptor complex of CD44-TLR4 (Garantziotis et al. 2010; Taylor et al. 2007) activating an intracellular signaling cascade including MyD88, TIRAP, and NF-κB (Garantziotis et al. 2010; Li et al. 2011) that result in increased mRNA expression of *pro-IL-1β* in alveolar macrophages. Either ozone or hyaluronan results in cleavage and activation of pro-caspase1 resulting in activation of the Nlrp3-inflammasome complex. Finally, the activated inflammasome complex results in cleavage of pro-IL-1β into active soluble IL-1β. Our results suggest that active IL-1β contributes to the complex signaling network required for pro-inflammatory signaling and development of AHR. Together these new findings provide additional support of a dominant role of genes of innate immunity in non-allergic reactive airways disease. Understanding the mechanisms that contribute to ozone-induced functional phenotypes in the airway can provide fundamental insight into the pathogenesis of AHR, a defining phenotype of clinical asthma. Our results establish a

central role for the Nlrp3-inflammasome in both the induction of AHR and production of pro-inflammatory cytokines. Our observations support that airway therapy targeted towards local deactivation of the Nlrp3-inflammasome may provide benefit to some patients with reactive airways disease.

References

Al-Hegelan M, Tighe RM, Castillo C, Hollingsworth JW. 2011. Ambient ozone and pulmonary innate immunity. Immunol Res 49(1-3):173-191.

Backus GS, Howden R, Fostel J, Bauer AK, Cho HY, Marzec J, et al. 2010. Protective role of interleukin-10 in ozone-induced pulmonary inflammation. Environ Health Perspect 118(12):1721-1727.

Barker BR, Taxman DJ, Ting JP. 2011. Cross-regulation between the IL-1beta/IL-18 processing inflammasome and other inflammatory cytokines. Curr Opin Immunol 23(5): 591-597.

Bauernfeind F, Bartok E, Rieger A, Franchi L, Nunez G, Hornung V. 2011. Cutting Edge: Reactive Oxygen Species Inhibitors Block Priming, but Not Activation, of the NLRP3 Inflammasome. J Immunol 187(2):613-617.

Bell M, McDermott A, Zeger S, Samet J, Dominici F. 2004. Ozone and short-term mortality in 95 US urban communities, 1987-2000. JAMA 292(19):2372-2378.

Bell ML, Peng RD, Dominici F. 2006. The exposure-response curve for ozone and risk of mortality and the adequacy of current ozone regulations. Environ Health Perspect 114(4):532-536.

Berman JD, N. Fann, J.W. Hollingsworth, K.E. Pinkerton, W.N. Rom, A.M. Szema, P.N. Breysse, R.H. White, F. C. Curriero. 2012. Health Benefits from Large Scale Ozone Reduction in the United States. Environ Health Perspect [ahead of print, PMID:22809899].

Caceres AI, Brackmann M, Elia MD, Bessac BF, del Camino D, D'Amours M, et al. 2009. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. Proc Natl Acad Sci U S A 106(22):9099-9104.

Chang HH, Zhou J, Fuentes M. 2010. Impact of climate change on ambient ozone level and mortality in southeastern United States. Int J Environ Res Public Health 7(7):2866-2880.

Dentener MA, Vernooy JH, Hendriks S, Wouters EF. 2005. Enhanced levels of hyaluronan in lungs of patients with COPD: relationship with lung function and local inflammation. Thorax 60(2):114-119.

Foster WM, Brown RH, Macri K, Mitchell CS. 2000. Bronchial reactivity of healthy subjects: 18-20 h postexposure to ozone. J Appl Physiol 89(5):1804-1810.

Garantziotis S, Li Z, Potts EN, Kimata K, Zhuo L, Morgan DL, et al. 2009. Hyaluronan mediates ozone-induced airway hyperresponsiveness in mice. J Biol Chem 284(17):11309-11317.

Garantziotis S, Li Z, Potts EN, Lindsey JY, Stober VP, Polosukhin VV, et al. 2010. TLR4 is Necessary for Hyaluronan-mediated Airway Hyperresponsiveness After Ozone Inhalation. Am J Respir Crit Care Med 181(7):666-675.

Guarda G, Zenger M, Yazdi AS, Schroder K, Ferrero I, Menu P, et al. 2011. Differential expression of NLRP3 among hematopoietic cells. J Immunol 186(4):2529-2534.

Hatch GE, Slade R, Harris LP, McDonnell WF, Devlin RB, Koren HS, et al. 1994. Ozone dose and effect in humans and rats. A comparison using oxygen-18 labeling and bronchoalveolar lavage. Am J Respir Crit Care Med 150(3):676-683.

Hoffman HM, Brydges SD. 2011. Genetic and molecular basis of inflammasome-mediated disease. J Biol Chem 286(13):10889-10896.

Hollingsworth JW, Cook DN, Brass DM, Walker JK, Morgan DL, Foster WM, et al. 2004. The Role of Toll-like Receptor 4 in Environmental Airway Injury in Mice. Am J Respir Crit Care Med 170(2):126-132.

Ito K, De Leon SF, Lippmann M. 2005. Associations between ozone and daily mortality: analysis and meta-analysis. Epidemiology 16(4):446-457.

Jin C, Flavell RA. 2010. Molecular mechanism of NLRP3 inflammasome activation. J Clin Immunol 30(5):628-631.

Johnston RA, Mizgerd JP, Flynt L, Quinton LJ, Williams ES, Shore SA. 2007. Type I interleukin-1 receptor is required for pulmonary responses to subacute ozone exposure in mice. Am J Respir Cell Mol Biol 37(4):477-484.

Kehrl HR, Vincent LM, Kowalsky RJ, Horstman DH, O'Neil JJ, McCartney WH, et al. 1987. Ozone exposure increases respiratory epithelial permeability in humans. Am Rev Respir Dis 135(5):1124-1128.

Kim CS, Alexis NE, Rappold AG, Kehrl H, Hazucha MJ, Lay JC, et al. 2011. Lung function and inflammatory responses in healthy young adults exposed to 0.06 ppm ozone for 6.6 hours. Am J Respir Crit Care Med 183(9):1215-1221.

Kleeberger SR, Reddy SP, Zhang LY, Cho HY, Jedlicka AE. 2001. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. Am J Physiol Lung Cell Mol Physiol 280(2):L326-333.

Koren HS, Devlin RB, Graham DE, Mann R, McGee MP, Horstman DH, et al. 1989. Ozone-induced inflammation in the lower airways of human subjects. Am Rev Respir Dis 139(2):407-415.

Li Z, Potts-Kant EN, Garantziotis S, Foster WM, Hollingsworth JW. 2011. Hyaluronan signaling during ozone-induced lung injury requires TLR4, MyD88, and TIRAP. PLoS One 6(11): e27137.

Li Z, Potts EN, Piantadosi CA, Foster WM, Hollingsworth JW. 2010. Hyaluronan fragments contribute to the ozone-primed immune response to lipopolysaccharide. J Immunol 185(11):6891-6898.

Liang J, Jiang D, Jung Y, Xie T, Ingram J, Church T, et al. 2011. Role of hyaluronan and hyaluronan-binding proteins in human asthma. J Allergy Clin Immunol 128(2):403-411 e403.

Moore PE, Lahiri T, Laporte JD, Church T, Panettieri RA, Jr., Shore SA. 2001. Selected contribution: synergism between TNF-alpha and IL-1 beta in airway smooth muscle cells: implications for beta-adrenergic responsiveness. J Appl Physiol 91(3):1467-1474.

Park JW, Taube C, Swasey C, Kodama T, Joetham A, Balhorn A, et al. 2004. IL-1 Receptor Antagonist Attenuates Airway Hyperresponsiveness Following Exposure to Ozone. Am J Respir Cell Mol Biol 30:30.

Peden DB. 2011. The role of oxidative stress and innate immunity in O(3) and endotoxin-induced human allergic airway disease. Immunol Rev 242(1):91-105.

Que LG, Stiles JV, Sundy JS, Foster WM. 2011. Pulmonary Function, Bronchial Reactivity, and Epithelial Permeability are Response Phenotypes to Ozone and Develop Differentially in Healthy Humans. J Appl Physiol. 2011 Sep;111(3):679-87.

Sahu S, Lynn WS. 1978. Hyaluronic acid in the pulmonary secretions of patients with asthma. Biochem J 173(2):565-568.

Samet JM. 2011. The Clean Air Act and health--a clearer view from 2011. N Engl J Med 365(3):198-201.

Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, Grant EP, et al. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity 24(3):317-327.

Taylor KR, Yamasaki K, Radek KA, Nardo AD, Goodarzi H, Golenbock D, et al. 2007. Recognition of Hyaluronan Released in Sterile Injury Involves a Unique Receptor Complex Dependent on Toll-like Receptor 4, CD44, and MD-2. J Biol Chem 282(25):18265-18275.

Ting JP, Willingham SB, Bergstralh DT. 2008. NLRs at the intersection of cell death and immunity. Nat Rev Immunol 8(5):372-379.

Verhein KC, Jacoby DB, Fryer AD. 2008. IL-1 receptors mediate persistent, but not acute, airway hyperreactivity to ozone in guinea pigs. Am J Respir Cell Mol Biol 39(6):730-738.

Wiester MJ, Tepper JS, King ME, Menache MG, Costa DL. 1988. Comparative study of ozone (O3) uptake in three strains of rats and in the guinea pig. Toxicol Appl Pharmacol 96(1):140-146.

Williams AS, Leung SY, Nath P, Khorasani NM, Bhavsar P, Issa R, et al. 2007. Role of TLR2, TLR4, and MyD88 in murine ozone-induced airway hyperresponsiveness and neutrophilia. J Appl Physiol 103(4):1189-1195.

Williams AS, Nath P, Leung SY, Khorasani N, McKenzie AN, Adcock IM, et al. 2008. Modulation of ozone-induced airway hyperresponsiveness and inflammation by interleukin-13. Eur Respir J 32(3):571-578.

Wu ZX, Barker JS, Batchelor TP, Dey RD. 2008. Interleukin (IL)-1 regulates ozone-enhanced tracheal smooth muscle responsiveness by increasing substance P (SP) production in intrinsic airway neurons of ferret. Respir Physiol Neurobiol 164(3):300-311.

Yamasaki K, Muto J, Taylor KR, Cogen AL, Audish D, Bertin J, et al. 2009. NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury. J Biol Chem 284(19):12762-12771.

Figure Legends

Figure 1. Biologic response to a time course after ozone exposure. Using wild type mice, we identified a significant increase in cellular inflammation (**A-C**), BAL protein (**D**) and BAL HA production (**E**) at 24, 48 and 72 hours after exposure to ozone (1 ppm x 3 h) compared with naive(unexposed mice). No increase in BAL total cell, macrophages or the level of BAL HA were detected at 3, 6, 12 hours after exposure; neutrophil in BAL started to increase at 12 hours, peaked at 24 hours and kept the highest level until 72 hours after ozone. (n=5~8; **p*<0.05, vs. naive; # *p*<0.05, vs. 3h; Δ *p*<0.05, vs. 6h; φ *p*<0.05, vs. 12h; ω *p*<0.05, vs. 24h; δ *p*<0.05, vs. 72h). (**F**) IL-1β mRNA in alveolar macrophages was upregulated at 6 hours and maintained throughout the 72 hours time course (n=5~8; **p*<0.05, vs. naive; # *p*<0.05, vs. 3h). (**G**) IL-18 mRNA in alveolar macrophages increased from 24 hours and peaked at 72 hours after exposure. (n=5~8; **p*<0.05, vs. naive; #*p*<0.05, vs. 3h; Δ*p*<0.05, vs. 6h; φ*p*<0.05, vs. 12h; δ*p*<0.05, vs. 72h). Data presented as mean ± SEM and are representatives from two similar experiments.

Figure 2. The role of Nlrp3-inflammasome in airway responses to ozone exposure. Wild type mice and inflammasome deficient mice were exposed to ozone (1ppm x 3h). Airway contractility to methacholine challenge, cell infiltration and BAL protein level were measured at 24 hours after exposure. **(A)** caspase1, ASC and Nlrp3 are necessary for the development of ozone-induced airway hyperresponsiveness (AHR) (n=5, **p*<0.05); **(B)** Total cells, macrophages and neutrophils in bronchoalveolar lavage (BAL) from wild type mice with ozone exposure were all higher than those from caspase1, ASC and Nlrp3 deficient mice (n=5, **p*<0.05); **(C)** The level of BAL protein was higher with ozone exposure, and completely dependent on caspase1and

ASC, partially dependent on Nlrp3 (n=5, *p<0.05). Data presented as mean ± SEM and are representatives from two similar experiments.

Figure 3. **IL-1β, IL-18, and hyaluronan 24 hours after ozone exposure.** (**A-B**) The transcription levels of IL-1β and IL-18 in alveolar macrophages were upregulated at 24 hours after ozone exposure compared with air, and were not dependent on Nlrp3-inflammasome. (n=5, *p<0.05) (**C-D**) BAL IL-1β and IL-18 were increased at 24 hours after ozone and completely dependent on ASC and caspase-1, IL-1β depended on Nlrp3 partially, and IL-18 was dependent on Nlrp3 completely. (n=5, *p<0.05). **(E)** Wild type mice and inflammasome deficient mice were exposed to ozone (1ppm x 3h), 24 hours later, HA level in the BAL was measured. Ozone enhanced the level of soluble hyaluronan in BAL (n=5, *p<0.05), and the level of HA was not dependent on Nlrp3 inflammasomes. (**F**) Oropharygeal aspiration of hyaluronic acid-binding peptide (HABP) was performed immediately before ozone exposure, 24 hours later, the level of secreted IL-1β in BAL was detected. The increased BAL IL-1β by ozone exposure was partially decreased by HABP when compared with controls (n=5, *p<0.05, #p<0.05). Data presented as mean ± SEM and are representatives from two similar experiments.

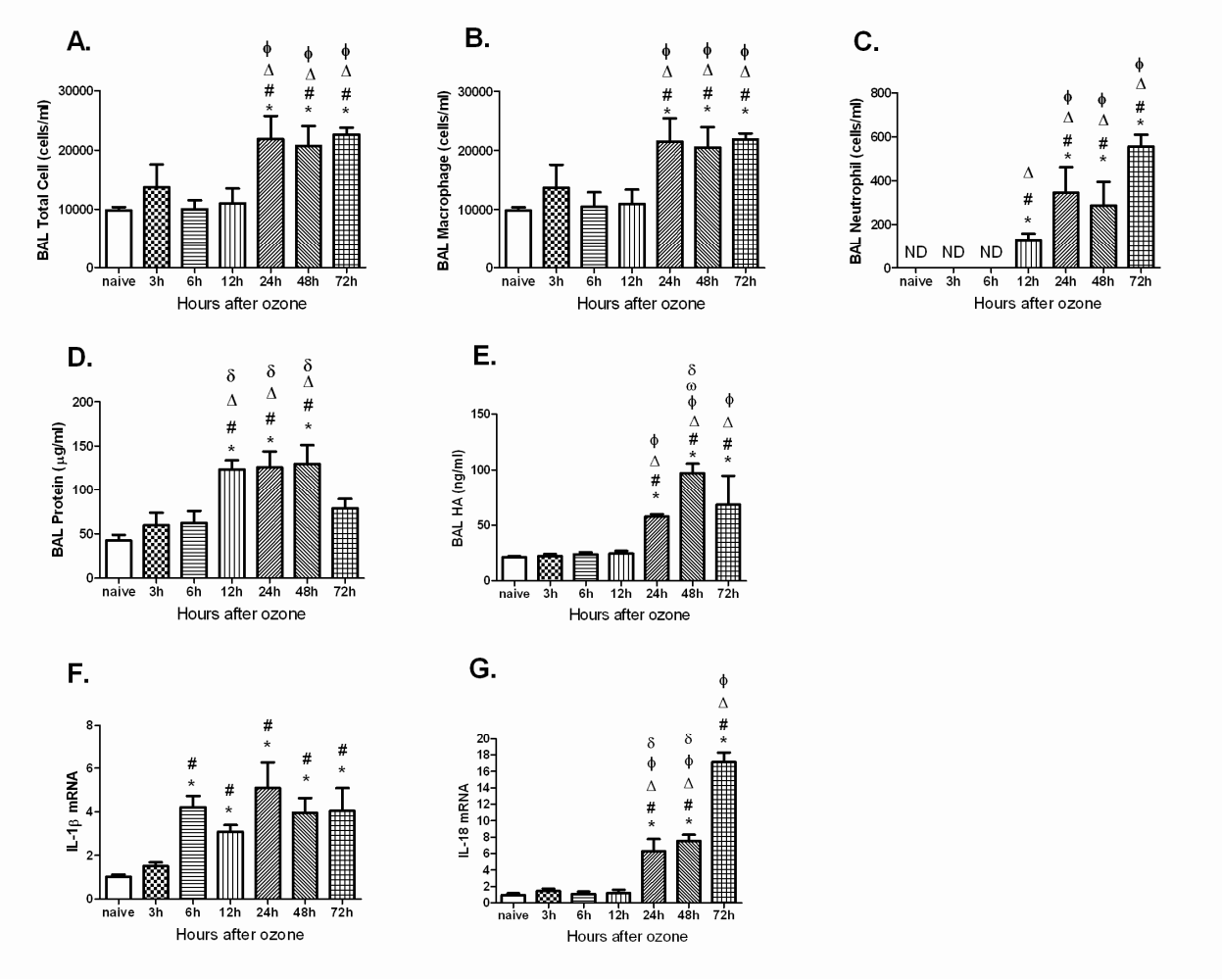
Figure 4. Biologic responses to a time course of hyaluronan treatment. 50 μl HA (25μg/mouse) or vehicle were instilled intratracheally into isoflurane-anesthetized wild type mice, biologic responses were observed at 1, 2 and 6 h after HA treatment. (**A-C**) There were no increase of BAL total cell, macrophages or neutrophil until 6 h after treatment (n=5, *p<0.05). (**D**) No increase in BAL protein was detected after HA treatment compared with vehicle at these three time points. (**E**) IL-1β mRNA in alveolar macrophages was upregulated 1h and 2h after HA treatment compared with vehicle (n=5, *p<0.05, #p<0.05), and reduced to close to baseline at 6h.

(**F**) There was no significant difference in the transcription level of IL-18 after HA treatment compared with vehicle at three time points. Data presented as mean ± SEM and are representatives from two similar experiments.

Figure 5. The role of Nlrp3 inflammasome in airway response to hyaluronan. Wild type mice and inflammasome deficient mice were challenged with short fragment hyaluronan (25μg/mouse). AHRs were detected at 2 h after treatment. When compared with wild type mice, caspase1, ASC and Nlrp3 deficient mice were protected from HA-induced airway hyperresponsivesness (AHR) (n=5, *p<0.05). Data presented as mean ± SEM and are representatives from two similar experiments.

Figure 6. Hyaluronan exposure enhances the level of IL-1β and cleavage of pro-caspase1 and pro-IL-1β. (**A**) The transcription level of IL-1β in alveolar macrophages was increased at 2 h after HA treatment compared with vehicle, and was not dependent on Nlrp3-inflammasome. (n=5, *p<0.05). (**C**) IL-1β in BAL from wild type mice was higher than that from Nlrp3-inflammasome deficient mice with HA treatment (n=5, *p<0.05). (**B**) IL-18 mRNA expression in alveolar macrophages or **(D)** the protein level of secreted IL-18 in BAL were not increased after direct challenge to hyaluronan fragments. Next, hyaluronan challenge results in cleavage of pro-caspase1 and cleavage of pro-IL-1β in WT mice but not in ASC-/-, Nlrp3-/-, or caspase1-/- mice. Mice were challenged to vehicle or hyaluronan fragments. After 2 hours, alveolar macrophages were isolated and protein was extracted (n=5/mice per group, samples pooled for analysis). Western blot analysis for **(E)** cleaved caspase1 and **(F)** cleaved IL-1β identifies cleavage only in WT mice exposed to hyaluronan fragments. Data presented as mean ± SEM and are representatives from two similar experiments.

Figure 1.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30000
20000
10000
0
naive 3h 6h 12h 24h 48h 72h
Hours after ozone
B.
BAL Macrophage (cells/ml)
30000
20000
10000
0
naive 3h 6h 12h 24h 48h 72h
Hours after ozone
C.
BAL Neutrophil (cells/ml)
800
600
400
200
0
ND ND ND
naive 3h 6h 12h 24h 48h 72h
Hours after ozone
D.
BAL Protein (μg/ml)
200
150
100
50
0
naive 3h 6h 12h 24h 48h 72h
Hours after ozone
E.
BAL HA (ng/ml)
150
100
50
0
naive 3h 6h 12h 24h 48h 72h
Hours after ozone
F.
IL-1β mRNA
8
6
4
2
0
naive 3h 6h 12h 24h 48h 72h
Hours after ozone
G.
IL-18 mRNA
20
18
16
14
12
10
8
6
4
2
0
naive 3h 6h 12h 24h 48h 72h
Hours after ozone

Figure 2.

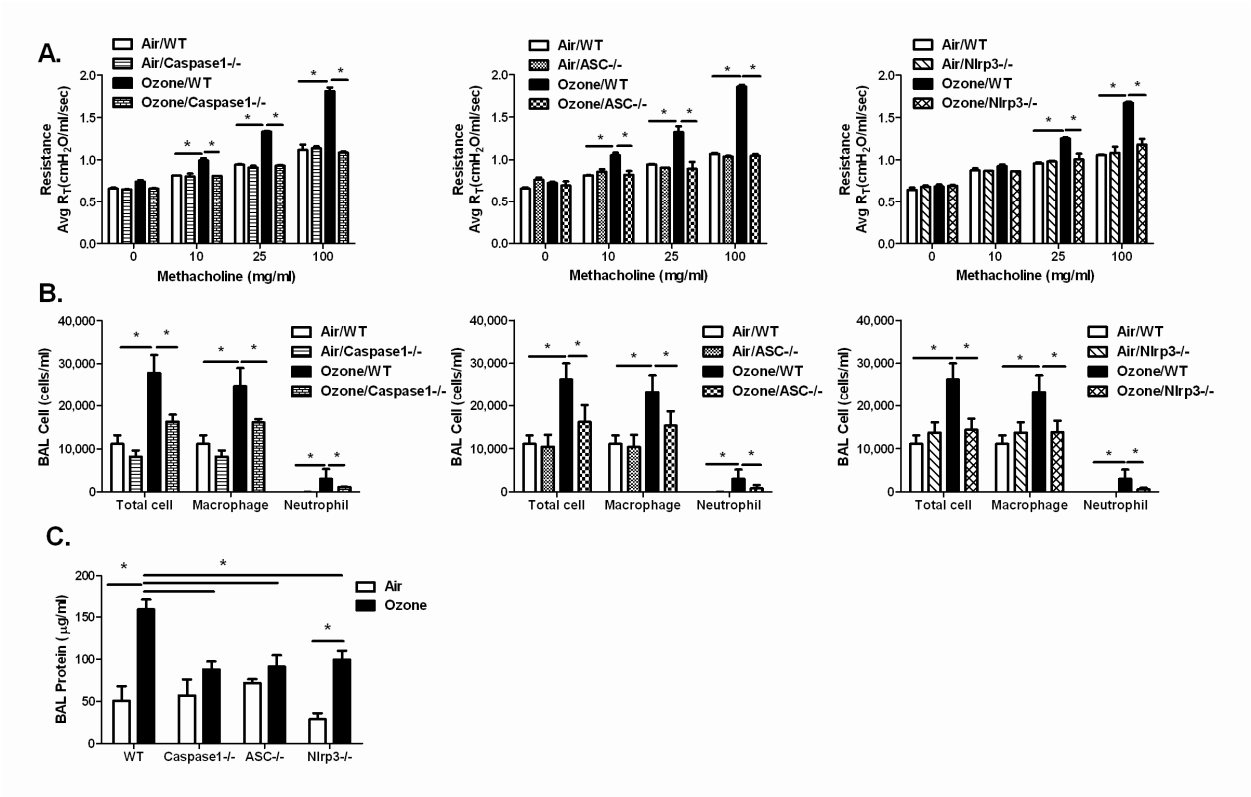
A.
Air/WT
Air/Caspase1-/-
Ozone/WT
Ozone/Caspase1-/-
Resistance
Avg R_T(cmH2O/ml/sec)
Methacholine (mg/ml)
Air/WT
Air/ASC-/-
Ozone/WT
Ozone/ASC-/-
Air/WT
Air/Nlrp3-/-
Ozone/WT
Ozone/Nlrp3-/-
B.
BAL Cell (cells/ml)
Total cell
Macrophage
Neutrophil
C.
BAL Protein (μg/ml)
Air
Ozone
WT
Caspase1-/-
ASC-/-
Nlrp3-/-

Figure 3.

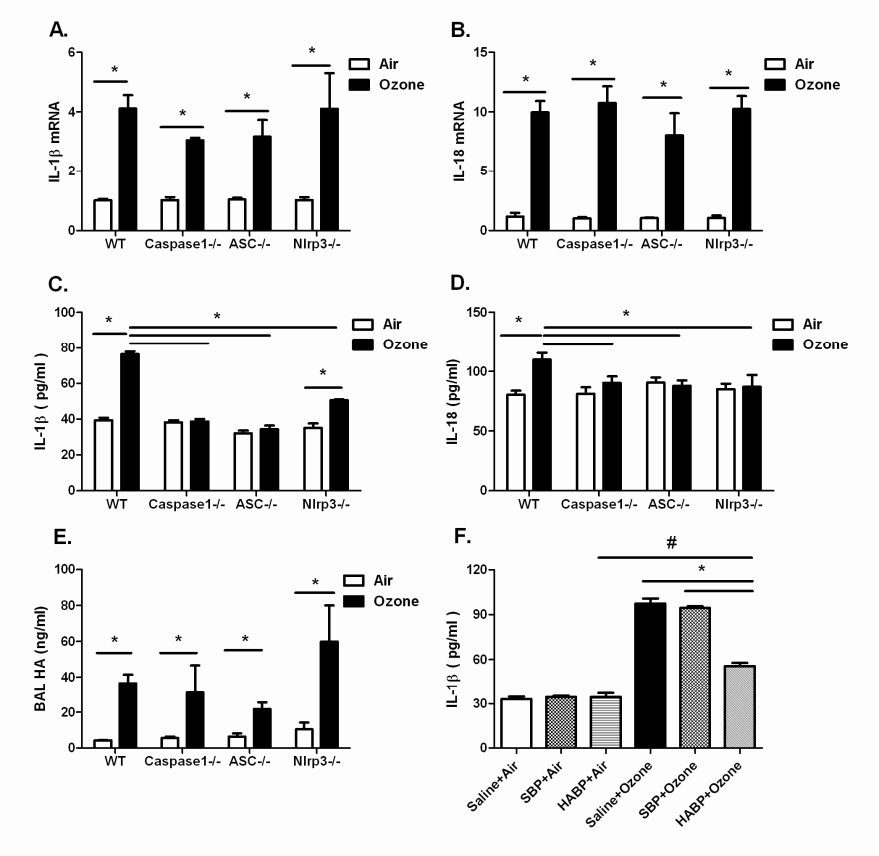
A.
IL-1β mRNA
Air
Ozone
WT
Caspase1-/-
ASC-/-
Nlrp3-/-
B.
IL-18 mRNA
C.
IL-1β (pg/ml)
D.
IL-18 (pg/ml)
E.
BAL HA (ng/ml)
F.
IL-1β (pg/ml)
Saline+Air
SBP+Air
HABP+Air
Saline+Ozone
SBP+Ozone
HABP+Ozone

Figure 4.

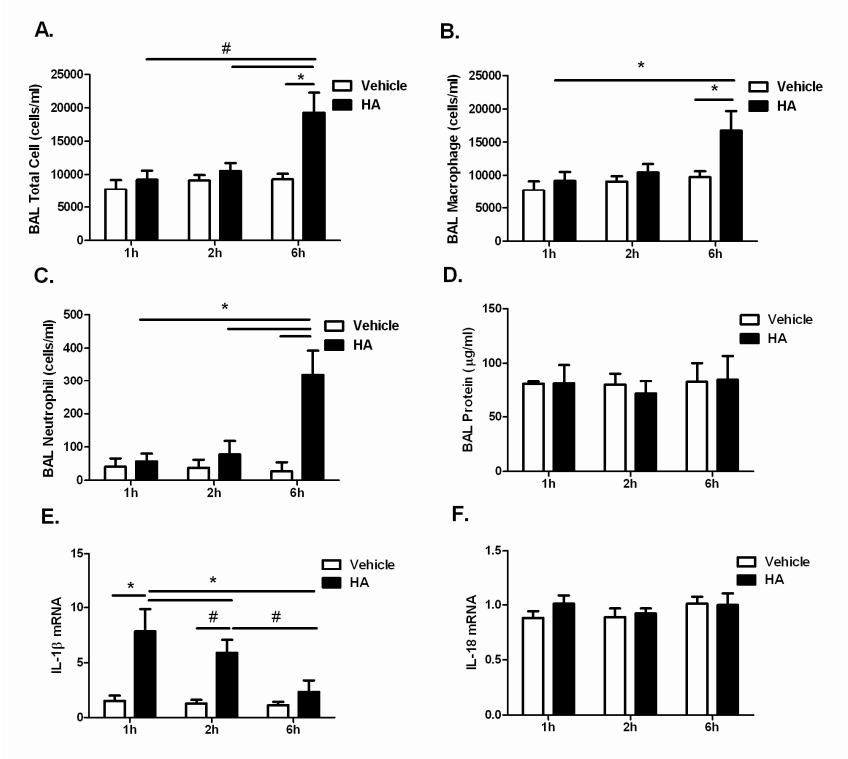
A.
BAL Total Cell (cells/ml)
25000
20000
15000
10000
5000
0
1h
2h
6h
#
*
Vehicle
HA
B.
BAL Macrophage (cells/ml)
*
*
C.
BAL Neutrophil (cells/ml)
500
400
300
200
100
*
D.
BAL Protein (μg/ml)
150
100
50
E.
IL-1β mRNA
15
10
5
*
*
#
#
F.
IL-18 mRNA
1.5
1.0
0.5
0.0

Figure 5.

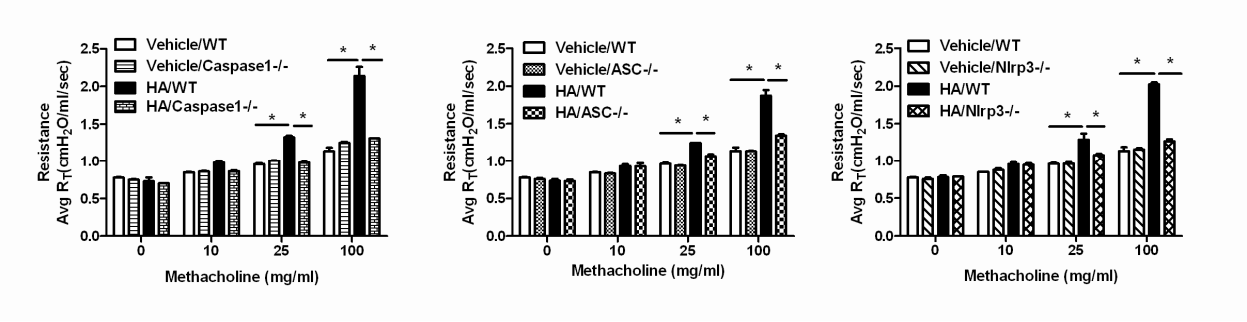

Figure 6.